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14. ABSTRACT
This project is developing an innovative method for regenerating bone that incorporates both temporal and spacial control of angiogenic and osteogenic genes, thereby allowing the patterning of vasculature and bone formation. The approach uses high intensity focused ultrasound (HIFU) and heat shock/ligand-dependent gene switches. Focused ultrasound generates localized hyperthermia to activate the heat shock response in a spatially restricted fashion, while ligand dependency improves stringency of expression. Substantial progress was made in all 3 task areas during year 1. 1. Generate adenoviral vectors for use in primary cells. Plasmid-based and retrovirus-based systems were developed for HIFU/heat shock inducible expression of VEGF165 and BMP2. 2. Establish stringency of therapeutic gene expression in transduced BMSCs. The plasmid expression systems developed under Task 1 were transfected into C3H10T1/2 mesenchymal cells. Tight control of luciferase, VEGF165 and BMP2 expression was demonstrated after heat-shock. 3. Determine the doses of HIFU required for inducing gene expression in vitro and in vivo. Optimal conditions for HIFU-induction were identified using a luciferase reporter gene stably integrated into C3H10T1/2 cells embedded in fibrin scaffolds. Using this approach, precisely patterned gene expression was achieved in vitro. HIFU-inducible VEGF and BMP2 expression were demonstrated. Lastly, heat shock induction of VEGF expression was demonstrated in vivo and shown to induce a robust subcutaneous angiogenic response.

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INTRODUCTION

This project is developing an innovative method for regenerating bone that incorporates both temporal and spacial control of angiogenic and osteogenic genes, thereby allowing the patterning of vasculature and bone. The approach uses high intensity focused ultrasound (HIFU) and heat shock/ligand-dependent gene switches. Focused ultrasound generates localized hyperthermia to activate the heat shock response in a spatially restricted fashion, while ligand dependency improves stringency of expression. We are developing vectors and cell lines that contain gene switches encoding vascular endothelial growth factor (VEGF) or bone morphogenetic protein 2 (BMP2) and determining the conditions and acoustic inputs necessary for activating these switches in vitro and in vivo. In addition, a 3D positioning system for raster scanning an ultrasound transducer is being developed; this will allow high resolution patterning of gene expression in engineered tissue constructs and in small animal models of osseous defects. During the past year, substantial progress was made in all 3 task areas.

BODY

Project Task 1: Generate adenoviral vectors for use in primary cells.

a. Revise current animal use protocol to include proposed in vivo HIFU experiments.

A revised animal use protocol has been approved by the U-M University Committee on Use and Care of Animals for performing the proposed in vivo experiments.

b. Construct and amplify adenoviruses encoding gene switches for bFGF and BMP2 transgenes.

Due to limitations in the size of the inserts accommodated by adenoviral backbones, we revised our strategy to use retroviruses that would both accommodate the switches and permit stable integration of transgenes into a target cell population. In addition, due to difficulties in achieving high secretion of bFGF from our expression vectors, we decided to pursue $VEGF_{165}$ as the angiogenic factor in our regeneration studies. **We currently have in-hand retroviral constructs containing $VEGF_{165}$ under the control of a heat- and rapamycin-inducible gene switch and BMP2 under the control of a heat- and mifepristone-inducible gene switch.** These vectors were amplified at the U-M Vector Core.

c. Verify that the gene circuits are packaged into the viruses.

Viral vectors containing inducible VEGF and BMP2 were verified by DNA sequenceing. However, we have not yet verified the functionality of these retroviral constructs.

Project Task 2: Establish stringency of therapeutic gene expression in transduced BMSCs.

a. Isolate and expand BMSCs from the long bones of 5-week-old C57BL6 male mice.

Due to difficulties in efficiently transfecting and transducing BMSCs, we developed an alternative strategy of transiently transfecting cells of the mesenchymal progenitor line C3H10T1/2 ("10T1/2") with the gene switches and establishing stable cell lines harboring the switches. These cells are readily transfected with exogenous DNAs using lipid-based reagents, can be expanded much more rapidly than primary BMSCs, and will thus serve as a useful platform for optimizing the activation of the gene switches with focused ultrasound. In parallel, we explored the use of alternative gene delivery methods (*e.g.*, electroporation) and vehicles for future studies with BMSCs.

b. Analyze expression of bFGF and BMP2 in BMSCs cotransduced with the adenoviruses prepared in Task 1.

Using cell lines containing stably-integrated gene switches for control of luciferase, BMP2 and $VEGF_{165}$, we showed that high levels of fLuc, VEGF and BMP2 expression can be achieved with very low background expression in the uninduced state (see figure 1 left, luciferase; figure 1, right, $VEGF_{165;}$ BMP2, not shown).

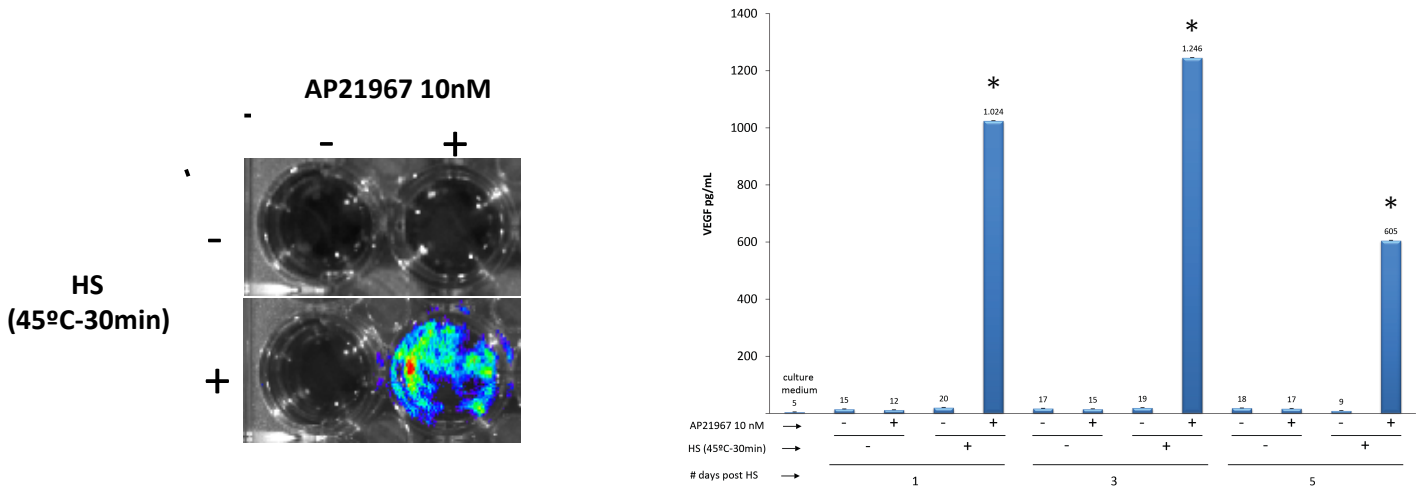

Figure 1. *Left.* Expression of the fLuc reporter gene in a 10T1/2 cell line harboring the heat shock- and rapamycin-dependent gene switch requires both a hyperthermic stimulus and the small molecule (in this case, AP21967, a synthetic analog of rapamycin). *Right*. Time course of VEGF secretion from a 10T1/2 cell line harboring the gene switch controlling expression of $VEGF_{165}$. Both heat shock ("HS") and AP21967 are required for expression of the regenerative molecule. * $p<0.01$ *vs.* unstimulated controls. Data are means + standard deviation for n = 3.

We have characterized the kinetics of activation and re-inducibility of 10T1/2 cells stably harboring the heat shock- and rapamycin-dependent gene switches. Upon exposure to the rapamycin analog AP29167 and a hyperthermic stimulus (incubation at 45C for 30min), the cells exhibited a peak in transgene expression at 24h post-heat shock and subsequent decay to baseline by 7 days post-heat shock (for VEGF see figure 2 left; for fLuc and BMP2 not shown but similar). The cells could also be re-induced to express the transgene by a second heat shock later in the experiment. Significantly, conditioned medium containing VEGF produced by activated cells demonstrated a significant proliferative effect ($p<0.05$) on human umbilical vein endothelial cells in vitro indicating that the secreted growth factor is bioactive (figure 2, right).

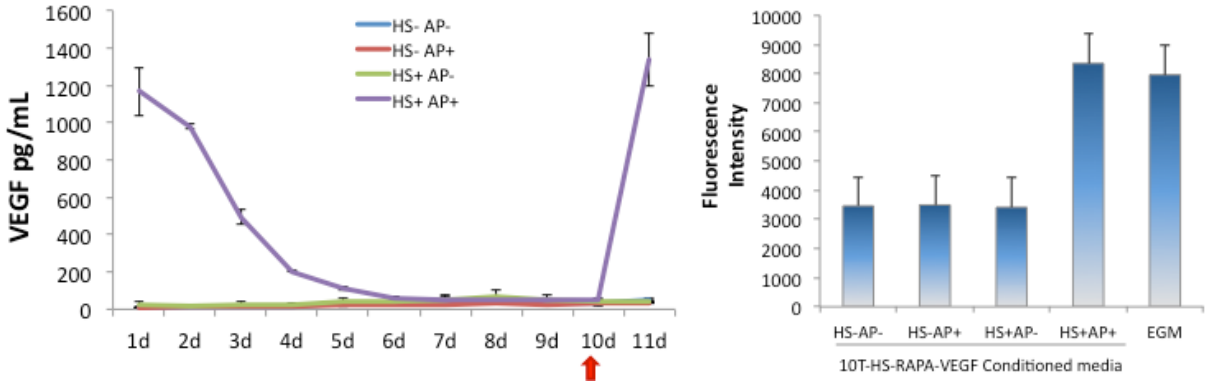

Figure 2. *Left.* 10T-HS-RAPA-VEGF cells suspended in fibrin scaffolds were left untreated or exposed to 10 nM AP21967 (AP) and/or subjected to heat shock treatment at 45ºC/30min (HS). VEGF secreted to the medium was monitored every 24 h by enzyme-linked immunosorbent assay (ELISA). Ten days after induction, a second HS was delivered to the cells-fibrin constructs (red arrow) in the absence or presence of AP. Reactivation of hVEGF165-gene switch was detected exclusively in cells that received the heterodimerizer. *Right.* Proliferative response of human umbilical vein endothelial cells (HUVECs) to conditioned medium of 10T-HS-RAPA-VEGF cells 24 h after being left untreated or exposed to 10 nM AP21967 (AP) and/or subjected to heat shock treatment at 45ºC/30min (HS). EGM (Endothelial cell Growth Medium) was used as a proliferation control for HUVECs. Cell number was measured by incubating cells with the alamar blue substrate, which undergoes conversion to a fluorescent product within mitochondria of live cells; thus, cell number is proportional to fluorescence intensity.

The VEGF-expressing cells could also be activated in vivo by exposure to hyperthermia and systemic administration of rapamycin. The cells were suspended in a solution of fibrin precursors (10mg/mL fibrinogen & 2U/mL thrombin) and injected into a subcutaneous space on the backs of syngeneic (C3H) immunocompetent mice. The fibrin polymerized in situ to form a cell-scaffold construct of ~400uL in volume. The cells were activated in vivo by submerging a small region of each mouse's back in a 45C water bath for 20min. The mice were then returned to their cages and injected with 1mg/kg rapamycin every 2 days for up to one week. We observed a robust vascular response at the gross and histologic levels in implants exposed to heat shock and rapamycin characterized by the formation of red blood cell-filled blood vessels (fig 3).

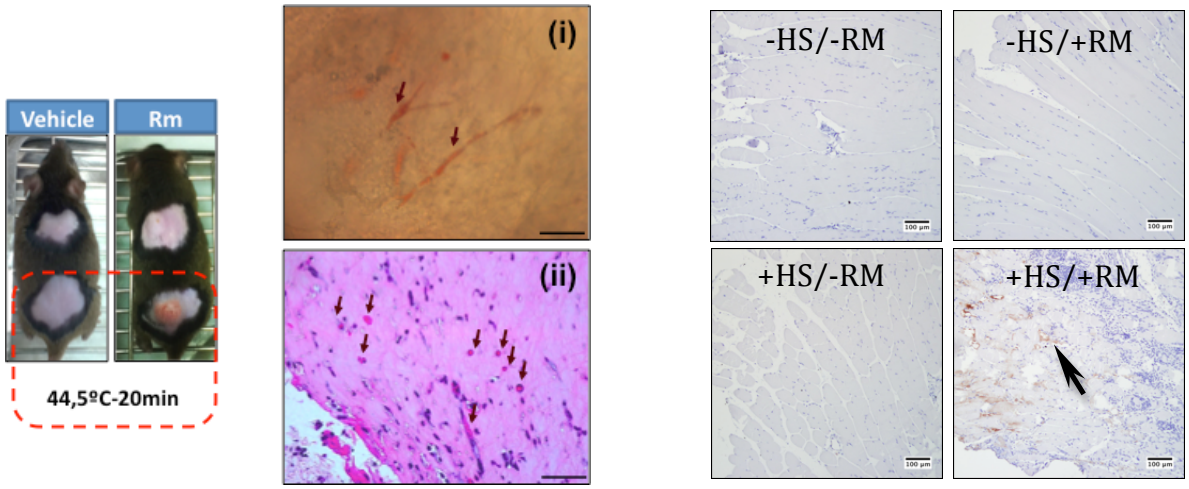

Figure 3. 10T-HS-RAPA-VEGF Cells-fibrin constructs were implanted on the backs of C3H mice. **(Left)** External appearance of implants 5 days after HS stimulation and administration of vehicle or rapamycin (Rm) every two days. Microscopic images of the activated implant unstained (i) or Hematoxylin&Eosin stained (ii) showed functional vasculature associated with bioactivity of transgenic VEGF secretion promoted by the gene switch. Arrows: red blood cell-filled neovessel within the implant. Scale bar=100 mm. **(Right)** Immunohistochemical staining for the vascular marker von Willebrand Factor (vWF) in skeletal muscle adjacent to the implants. vWF staining (arrow) was only detected in implants exposed to both the hyperthermic stimulus and rapamycin. Sections were counterstained with hematoxylin. Scale bar=100 um.

Project Task 3: Determine the doses of HIFU required for inducing gene expression in vitro and in vivo.

a. *Examine expression of growth factors in cell-scaffold constructs subjected to various doses of pulsed wave HIFU in vitro.*

We have successfully demonstrated ultrasound-induced, spatially-restricted expression of reporter and growth factor transgenes in vitro. In the initial phases of the project we used previously described HeLa cell lines harboring either a heat shock- and mifepristone-dependent switch (referred to as line J55 [1]) or a simpler heat shock-dependent switch (referred to as line M1 [2]) driving expression of a firefly luciferase ("*f*Luc") reporter gene. The experimental approach involved suspending the cells in a scaffold at the bottom of a 6-well BioFlex culture dish and exposing the engineered tissues to focused ultrasound at a variety of amplitudes and durations in a custom apparatus (figure 4, left). After the ultrasound exposures the cultures were assayed for fLuc expression by bioluminescent imaging (BLI) (figure 4, right). We observed spatially-restricted activation of M1 cells suspended in an alginate scaffold following activation with focused ultrasound, but these experiments revealed some of the difficulties of working with alginate (*e.g.*, rapid and heterogeneous polymerization, frank disruption of the scaffold at the focus) and motivated investigation of other scaffolding materials.

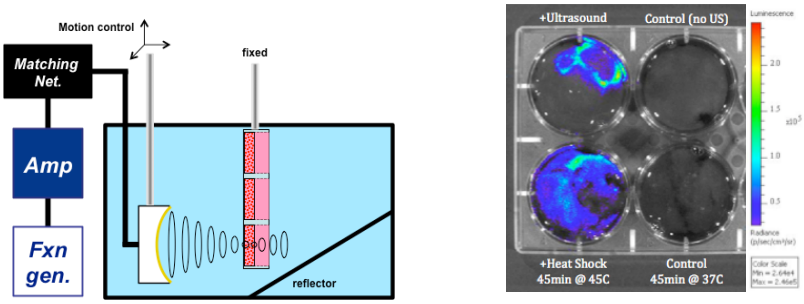

Figure 4. *Left.* Schematic of the experimental setup for applying focused ultrasound to cell-scaffold constructs. The sealed culture dish was immersed in a bath of 37°C degassed water and the transducer was positioned by computerized motion control. In some studies the temperature at/near the focus was monitored by a needle-type thermocouple. *Right*. Representative BLI data demonstrating activation of M1 cells in an alginate scaffold following ultrasound exposure (upper left well) or incubation on a 45°C water bath for 45min (lower left well).

Preliminary studies indicated that pulsed wave focused ultrasound was inefficient at heating the cell-scaffold constructs, so subsequent experiments were performed with continuous wave (CW) focused ultrasound. This greatly reduced the parameter space for optimizing ultrasound doses. In type I collagen scaffolds **we observed consistent amplitude- and exposure time-dependent activation of cell line M1** (figure 5, left). Similar patterns of activation were observed for HeLa cells harboring the full heat- and rapamycin-dependent switch (cell line "#73") suspended in a fibrin scaffold (figure 3 center). We found that for ultrasound exposures ≥ 10' the collagen scaffolds underwent substantial deformation. Fibrin scaffolds are more robust and appear to maintain their geometry under even longer and higher amplitude ultrasound exposures. A 10T1/2 cell line harboring the heat- and rapamycin-dependent switch controlling expression of a $VEGF_{165}$ transgene also exhibits tight control and high, exposure time-dependent levels of expression in groups exposed to rapamycin and focused ultrasound or bulk hyperthermia (figure 5, right panels) in the context of a fibrin scaffold.

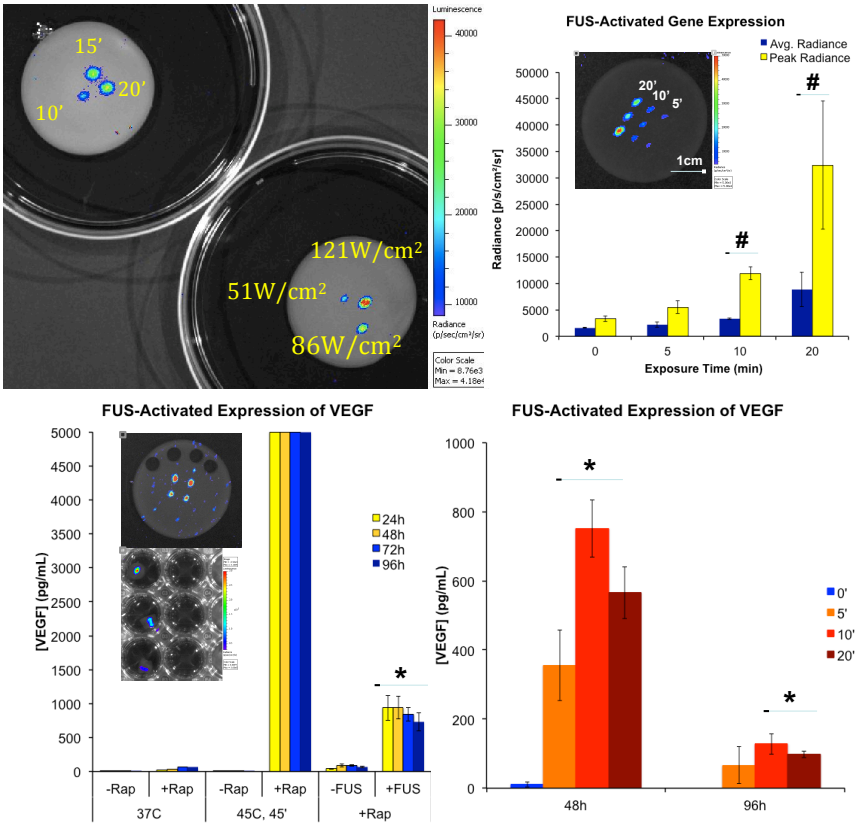

Figure 5. *Upper Left.* Exposure time- and amplitude-dependent expression of the fLuc transgene in M1 cells (harboring a heat shock-inducible switch) suspended in a collagen scaffold. The intensity and radius of the activated zone increases with both parameters. *Upper Right*. Similar behavior was observed in #73 cells (harboring a heat shock- and rapamycin-inducible switch) in a fibrin scaffold, with peak and average radiances serving as quantitative measures of fLuc expression. # p<0.05 *vs.* unstimulated control regions of the construct. Inset shows BLI imaging *Lower panels*. A 10T1/2 cell line containing the switch driving expression of $VEGF_{165}$ shows similar stringency and exposure time-dependent expression of the transgene that persists for at least 96h. Insets show BLI images of ultrasound-induced transgene expression.* p<0.05 vs. unstimulated regions of the construct. Data are means +/- SD for n = 4.

We also investigated the effects of ultrasound exposure on necrosis and apoptosis in the focal zone of the cell-scaffold constructs using fluorescent labeling of the actin network and annexin V labeling, respectively, and examined the microstructure of the fibrin scaffold in these zones with fluorescently tagged fibrinogen. By confocal microscopy we detected clear regions of cell death, denoted by rounded-up cell morphology and positive annexin V labeling, but did not observe disruption of the scaffold material and the fraction of cell death

for exposure times ≤10min appears to be low. Indeed, expression of the transgenes in the focal zone is detected for at least 96h after ultrasound exposure, and from this result we can conclude that a substantial fraction of cells in the focal zone survives.

b. Using a computer-controlled x-y-z stage to manipulate the position of the HIFU transducer, pattern transgene expression in cell-scaffold constructs in vitro.

The 3D positioning system described in figure 1 has been useful for optimizing the acoustic parameters required for activating cells in our engineered tissue constructs, and through sequential ultrasound exposures at multiple points **we were able to generate complex 3D patterns of transgene expression**. Ultrasound-mediated induction of transgene expression could be localized with ~20 mm^3 resolution (~4 mm^2 axial, ~5 mm longitudinal) in three dimensions by moving the focus through the construct (Fig 6A). Cells engineered with the heat shock- and rapamycin-dependent switch driving expression of $VEGF_{165}$ also exhibited restricted activation following focal exposures to ultrasound. After exposure, the focal regions were excised and cultured for 24h; the resulting conditioned medium contained dramatically higher quantities of VEGF than controls taken from non-exposed regions (Fig 6B).

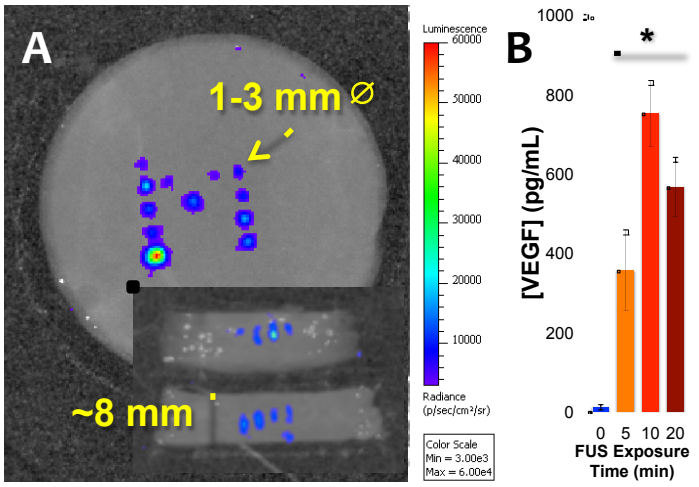

Figure 6. Complex 3D patterning of transgene expression using focused ultrasound. A block M (**A**) was patterned by multiple 5' exposures of focused ultrasound (I_{SPTA}=230 W/cm^2). The focus was moved into the scaffold with each step and the resulting depth-dependent activation is observed in cross sections shown in the inset. In **B** are preliminary data demonstrating FUS exposure time-dependent expression of $VEGF_{165}$. Little VEGF was detected in non-irradiated regions (0 min exp. time). Data are mean ± SD, n=3..

Although the current positioning system has been useful in these proof-of-principle studies, the apparatus requires submersion of the samples and is thus unsuitable for the proposed in vivo experiments. For this reason, we designed a 3D positioning system that eliminates the need for a large bath by implementing a coupling cone with the ultrasound transducer. The positioning system is an off-the-shelf gantry type system (Zaber Technologies) that we modified with custom fixtures to accommodate a focused ultrasound transducer and is controlled *via* custom LabView code (figure 7, left). The instrument is capable of smooth, high precision motion in 3 dimensions and will allow not only for ultrasound-induced rastering of transgene expression patterns in small animals, but also in engineered tissue constructs in vitro. We calibrated the acoustic output of the transducer in this configuration using a membrane hydrophone and processed the collected signals with custom MATLAB code to measure the dimensions, intensity, and distortion of the ultrasound beam near the focus (figure 7, right). Consistent with the expected beam profiles for our transducer, the focal volume gave a lateral width of ~1.1mm (full width at half maximum, FWHM) and an axial length of ~14mm FWHM. The output (spatial peak time average intensity, I_{SPTA}) from the transducer with the cone in place was somewhat lower (~25%) than measured previously in the free field, but the distortion remained quite low (<7%) even at peak intensities.

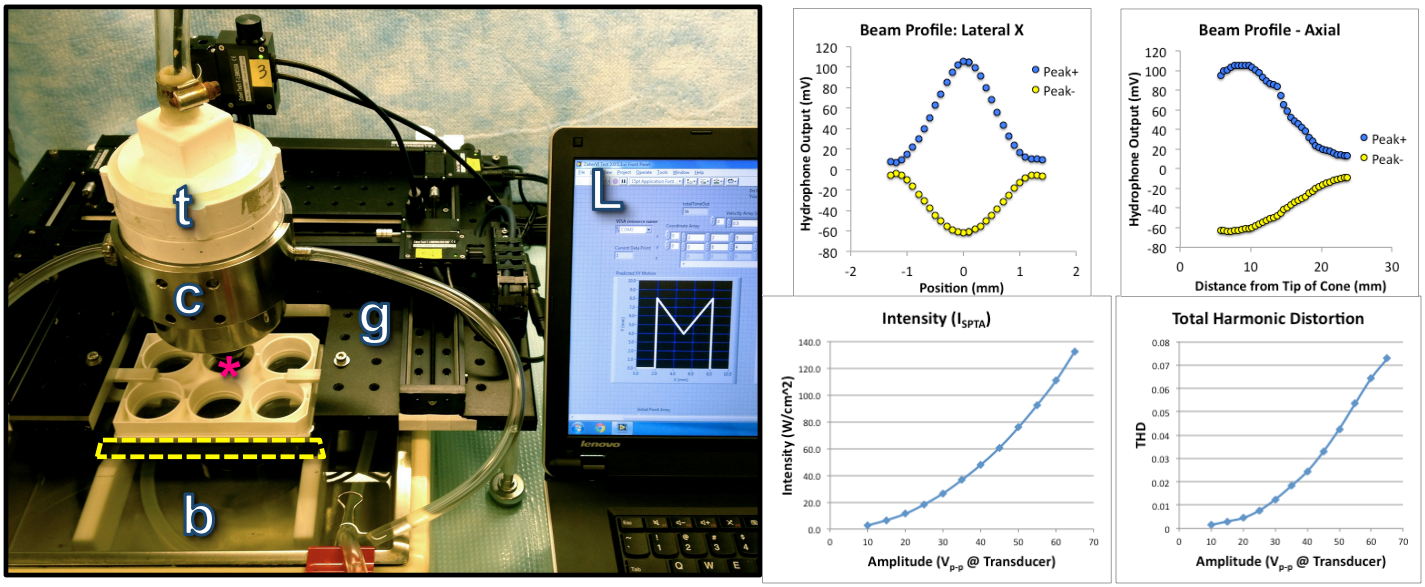

Figure 7. *Left*. Positioning system for raster scanning deposition of FUS in engineered tissues. (**L**) A LabVIEW virtual instrument controls the position of the FUS field through a 3-axis gantry (**g**). A water-filled cone (**c**) acoustically couples a FUS transducer (**t**) to the culture medium and cell-scaffold construct in a BioFlex® dish below (*). A 37C bath (**b**) maintains the temperature of the tissues, and an angled absorber/reflector (dashed yellow line) dissipates acoustic energy transmitted through the sample. *Right*. Lateral and axial beam profiles demonstrate a tightly focused peak in acoustic output (here indicated by hydrophone output; peak outputs correspond to +/- 3 MPa pressures at the focus). Intensity at the focus increases with voltage applied to the transducer, as does distortion.

c. Apply different doses of HIFU to subcutaneous implants of cell-scaffold constructs seeded with transduced BMSCs.

These studies are in the planning stages and will begin within the next 4-6 weeks.

Project Task 4: Investigate the use of transduced cell-scaffold constructs in an in vivo model of bone injury.

Planning of these studies will begin once the subcutaneous implant studies in Project task 3 have been completed.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of plasmids, retroviruses, and cell lines harboring a heat shock- and ligand-dependent gene switches encoding the fLuc reporter molecule, the osteogenic factor BMP2, and the angiogenic factor *h*VEGF$_{165}$.
- Characterization of activation kinetics and bioactivity of secreted transgene products in vitro and in vivo.
- Identification of acoustic parameters, including mode, amplitude, and duration of exposure, required for activating the gene switch with minimal loss of cell viability.
- Assembly of a computer controlled gantry-type 3D positioning system capable of raster scanning the focus of an ultrasound transducer through an engineered tissue (*i.e.*, cell-scaffold construct) or a wound bed (*i.e.*, in a small animal model of bone repair).

REPORTABLE OUTCOMES

The following cell lines have been generated in this project:

#73 – This is a line derived from the HeLa parent line that harbors a gene switch that regulates the expression of a firefly luciferase transgene and requires heat shock and rapamycin (or synthetic analog thereof) for activation.

Z3f15 - This is a line derived from the C3H10T1/2 parent line that harbors a gene switch that regulates the expression of a firefly luciferase transgene and requires heat shock and rapamycin (or synthetic analog thereof) for activation.
Z3V11 – This is a line derived from the C3H10T1/2 parent line that harbors a gene switch that regulates the expression of a hVEGF transgene and requires heat shock and rapamycin (or synthetic analog thereof) for activation.

Z3B28 - This is a line derived from the C3H10T1/2 parent line that harbors a gene switch that regulates the expression of a BMP2 transgene and requires heat shock and rapamycin (or synthetic analog thereof) for activation.

Results from this project have been reported in abstracts at 2 international conferences:

Wilson CG, Padilla F, Zhang M, Vilaboa N, Kripfgans O, Fowlkes JB, Franceschi RT. “Using Focused Ultrasound for the Spatiotemporal Control of Gene Expression in Engineered Tissue” *Annual Meeting of the Biomedical Engineering Society*, Hartford, CT (2011).

Wilson CG, Padilla F, Zhang M, Vilaboa N, Kripfgans O, Fowlkes JB, Franceschi RT. “Ultrasound-Induced Hyperthermia for the Spatio-Temporal Control of Gene Expression in Bone Repair” *International Society for Therapeutic Ultrasound*, New York, NY (2011).

The abstract for the ISTU meeting was also expanded for publication in the peer-reviewed conference proceedings.

This project also served as the basis for projects for two undergraduate engineering students through University of Michigan’s Undergraduate Research Opportunity Program (UROP). The two students presented their results at the 2011 UROP poster symposium held on the U-M campus in Ann Arbor, MI.

Lastly, preliminary data generated by this project served as the basis or an R01 application currently being evaluated by the NIH (Title: Control of Bone Regeneration Using Ultrasound-Activated Gene Expression).

CONCLUSION
We have made substantial progress in the preparation of reagents and cell lines as well as the design, assembly, and calibration of the hardware necessary for patterning transgene expression in vitro and in vivo. These accomplishments are significant because they represent the key components of a technology capable of previously unrealized spatiotemporal control over the expression of regenerative transgenes. Most developmental and wound healing processes are governed by a spatially- and temporally-restricted expression of morphogens, and the tools that we have developed over the last year should permit recapitulation of some of these patterns. We expect this technology to provide new avenues of fabricating tissue grafts ex vivo and a powerful means of guiding the regeneration process in vivo.

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[1] Vilaboa, N, Fenna, M, Munson, J, Roberts, SM and Voellmy, R. *Mol Ther* 12:2, 290-8 (2005)
[2] Martín-Saavedra, FM, Ruíz-Hernández, E, Boré, A, Arcos, D, Vallet-Regí, M and Vilaboa, N. *Acta Biomater* 6:12, 4522-31 (2010)

APPENDICES

1.) Abstract submitted to the 2011 Annual Meeting of the Biomedical Engineering Society (Hartford, CT)
2.) Proceedings paper currently in press from the 2011 International Symposium for Therapeutic Ultrasound (New York, NY)

Using Focused Ultrasound for the Spatiotemporal Control of Gene Expression in Engineered Tissues

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Introduction: High-volume tissue defects, common in cancer resections and traumatic injuries, are difficult to repair because there are few technologies or surgical approaches that recapitulate the spatially- and temporally-restricted expression of growth factors that drives the wound healing process. High intensity focused ultrasound (HIFU) is a technology capable of delivering energy to small volumes of tissue deep within the body, typically for mechanical and/or thermal ablation (*e.g.*, of cancers). We propose the use of HIFU for locally activating cells engineered with heat/stress-sensitive gene switches to control the expression of regenerative molecules, such as growth factors; this approach could be used for *ex vivo* patterning of engineered tissues and/or non-invasive activation of implants *in vivo*. The objective was to identify HIFU exposure parameters and a cell-scaffold platform capable of sustaining the mild, sublethal hyperthermia needed to activate the gene switch, with minimal erosion of the cells and scaffold. In this study we used cells expressing a reporter gene, firefly luciferase (*f*Luc), to assess HIFU-dependent activation of the gene switch.

Materials and Methods: HeLa cells, stably expressing a gene switch that uses the promoter for heat shock protein 70b to drive expression of *f*Luc (Martín-Saavedra, *et al.*), were suspended in scaffolds composed of bovine fibrin, polymerized in 6-well Bioflex® culture dishes as 5mm-thick slabs, and cultured overnight in growth medium. All scaffold precursor solutions were degassed under vacuum prior to preparation. Cell-scaffold constructs were exposed to continuous wave 3.3MHz focused ultrasound from a custom transducer (focus at=101mm, f#1.33, axial/lateral FWHM=10/1 mm) in a tank filled with 37C degassed water. Some constructs were heated on a water bath for comparison with HIFU-induced activation of the gene switch. Later, the constructs were incubated with luciferin (*f*Luc substrate) and the bioluminescence was imaged and quantified on a Xenogen IVIS Spectrum. The next day, the constructs were fixed, stained for nuclei and filamentous actin, and examined by confocal microscopy.

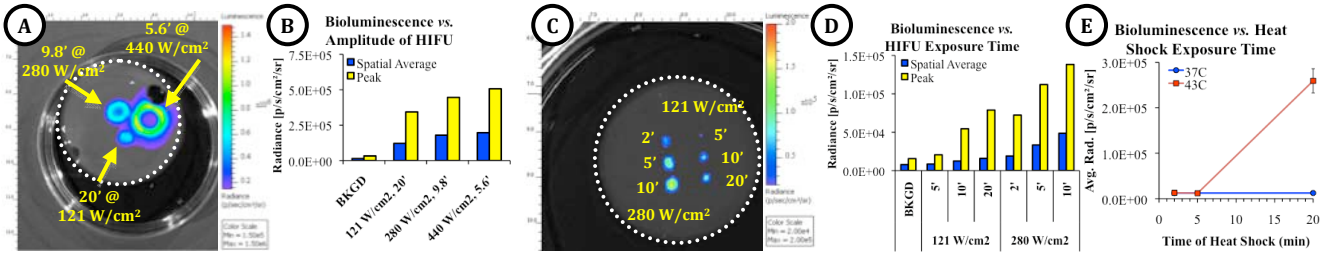

Figure 1. (A) Bioluminescent localization of HIFU-induced luciferase expression at 3 foci of similar energy deposition but different amplitudes. Quantification of radiances (means of 2 samples) is given in (B). (C, D) Strong bioluminescence is detected after a 2' exposure at 280 W/cm^2 (means of 2); this is short compared to heat shock on a water bath, which requires at least 5' for activation (E). Data in (E) are means ± SD, n = 3. Dotted lines in (A) and (C) indicate the perimeter (Ø=35mm) of the constructs. BKGD=background signal at non-exposed regions.

Results and Discussion: Constructs exhibited localized induction of *f*Luc activity with diameters and radiances that increased with amplitude (Fig 1A & B). Opaque lesions and loss of actin staining were evident at the central "well" of activation in 440W/cm^2 exposures (not shown), but not in regions irradiated with lower amplitudes. As little as 2' at 280 W/cm^2 activated *f*Luc expression ~4.5 fold over background levels, comparable to 20' at 121 W/cm^2 (Fig 1C & D), with little or no damage to the scaffold or cells. Interestingly, heating constructs on a water bath (no HIFU) required at least 5' to activate expression of *f*Luc (Fig 1E), suggesting that HIFU induces a stress additive to, or synergistic with, hyperthermia.

Conclusions: Our results demonstrate the HIFU-guided patterning of gene activation and a range of acoustic inputs for which we detected little or no acute damage to the cells and fibrin scaffold. Ongoing work addresses the mechanisms by which cells are activated by HIFU and development of switches for expression of growth factors.

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Reference: Martín-Saavedra FM, *et al.* Acta Biomater (2010) 6:12, 4522-31.

Ultrasound-Induced Hyperthermia for the Spatio-Temporal Control of Gene Expression in Bone Repair

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Abstract. Spatial and temporal control over the expression of growth/differentiation factors is of great interest for regeneration of bone, but technologies capable of providing tight and active control over gene expression remain elusive. We propose the use of focused ultrasound for the targeted activation of heat shock-sensitive expression systems in engineered bone. We report in vitro results with cells that express firefly luciferase (fLuc) under the control of a heat shock protein promoter. Cells were embedded in fibrin scaffolds and exposed to focused ultrasound, using a custom 3.3MHz transducer (focal length 4", f-number 1.33", focal dimension 1.2mm lateral FWHM) in CW mode for 2-20 minutes at intensities I_{SPTA}=120-440 W/cm^2. The kinetics of ultrasound-mediated activation of the cells was compared with that of strictly thermal activation. Bioluminescence imaging revealed fLuc expression in an area ≥2.5mm in diameter at the position of the ultrasound focus, and the diameter and intensity of the signal increased with the amplitude of the acoustic energy. We also found that ultrasound activated fLuc expression with substantially shorter exposures than thermal activation. Our results demonstrate the potential for focused ultrasound to selectively activate the expression of a gene of interest in an engineered tissue and suggest that focused ultrasound activates the heat shock pathway by a combination of thermal and non-thermal mechanisms.

Keywords: Focused Ultrasound, Hyperthermia, Gene Expression, Regeneration
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INTRODUCTION

High volume bone defects associated with traumatic injury and cancer resection are difficult to repair because there are no methods of guiding and sustaining the body's endogenous mechanisms of wound healing over such large dimensions. In fractures of bone that heal normally, wound healing proceeds *via* a sequence of events coordinated by the spatially- and temporally-restricted expression of growth factors such as bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF). Strategies for controlling the expression of growth factors in both time and space have the potential to recapitulate the endogenous healing process and promote regeneration of large bone defects. To-date, however, there are no technologies that provide stringent, active control over the expression of regenerative factors in 4 dimensions.

Ultrasound has demonstrated utility in accelerating the healing of bone fractures, but the mechanisms of therapeutic action remain unclear. Clinical doses of low intensity pulsed ultrasound (LIPUS) (*e.g.*, 20 min/day at 30mW/cm^2) promote differentiation of osteoblasts [1] and the expression of extracellular matrix proteins by chondrocytes [2]; LIPUS may also improve vascularization of the fracture site. Far less is known about the potential therapeutic effects of focused ultrasound in the context of regeneration. Biomedical applications of high intensity focused ultrasound (HIFU) have revolved primarily around the mechanical and thermal ablation of pathologic tissues or the non-invasive activation of cytotoxic genes in cancers [3]. Here we propose the application of this strategy to control the expression of specific regenerative molecules in engineered tissues. The objective of these studies was to identify focused ultrasound exposures that activate a heat shock gene switch with minimal cytotoxicity and minimal damage to a biopolymeric scaffold.

MATERIAL AND METHODS

HIFU Exposure

A custom-designed transducer was used to deliver focused ultrasound: center frequency 3.3 MHz, focal length 101mm, f-number 1.33, lateral FWHM 1.2mm, axial FWHM 10mm. Continuous wave (CW) excitation was performed by a function generator (HP33120A, Hewlett Packard, Palo Alto, CA) connected to an amplifier (A300, ENI, Rochester, NY). Acoustic output was calibrated using a membrane hydrophone (Sonic Industries, Seattle, WA) and is depicted in Figure 1.

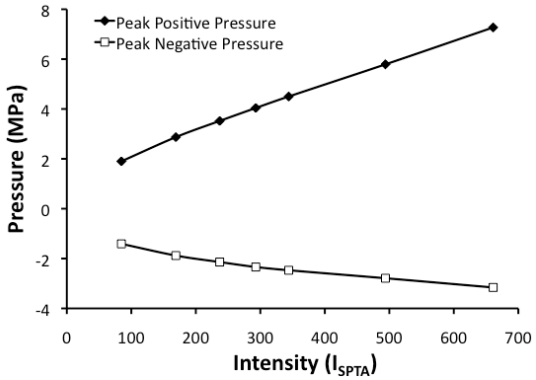

FIGURE 1. Peak positive and peak negative pressure as a function of the intensity at the focus of the HIFU transducer. Intensity was estimated as I_{SPTA} for a CW regime.

Cell Culture And Preparation Of Cell-Fibrin Constructs

We used a stable line of HeLa cells engineered to express firefly luciferase (fLuc) under the control of the promoter for heat shock protein (HSP) 70b [4]. The cells were propagated under chemical selection conditions to ensure persistence of the transgene. To determine the timecourse of activation by hyperthermia, cells were grown on tissue culture plastic or cultured in cell-fibrin constructs and heated by replacing the culture

medium with preheated medium and then placing the dishes on waterbaths at various temperatures for various durations.

Fibrin scaffolds were prepared by mixing a solution of fibrinogen (from bovine plasma, Sigma) in PBS with thrombin (from bovine plasma, Sigma). A suspension of cells in culture medium was then added at 40% v/v, and the constructs were cast in the wells of a Bioflex plate for polymerization at 37C. The final density of the fibrin scaffold was 10mg/mL (clottable protein) and a concentration of 2U/mL thrombin was used for all preparations. The final cell density was $5x10^5$ cells/mL. Cell-fibrin constructs were typically equilibrated overnight with complete medium consisting of Dulbecco's Modified Eagle Medium, fetal bovine serum 10% v/v, and penicillin, streptomycin, and gentamicin, prior to HIFU exposure.

Bioluminescence Imaging

Expression of fLuc was measured 3-6h after heating or exposure to HIFU by biochemical assay according to the manufacturer's instructions (Promega) or by bioluminescent imaging. For imaging studies the cell-scaffold constructs were incubated with 200μg/mL D-luciferin for 1h at 37C. The constructs were then imaged using an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA) instrument typically with low binning to achieve high resolutions. Measurements of spatially averaged and peak radiances were made in regions of interest using the LivingImage software (Caliper Life Sciences) and further processed and charted using Microsoft Excel. Images were modified by 3x3 smoothing and zooming for presentation purposes.

Confocal Microscopy

Cell-scaffold constructs were prepared for confocal microscopy 24h after HIFU exposures by rinsing in PBS, and fixing overnight in 10% neutral buffered formalin. Following fixation, the gels were stained with Hoechst 33528 (to stain nuclei) and AlexFluor488-phalloidin (to stain filamentous actin). The constructs were then rinsed with PBS and mounted for analysis. Images of fluorescence from the staining and reflected light from the scaffold material were captured on a inverted confocal microscope (SP5, Leica Microsystems, Wetzlar, Germany) with a fs-pulsed Ti:sapphire laser (Mai Tai Deep See, Spectra-Physics, Santa Clara, CA) for multiphoton excitation of the Hoechst 33258 and an argon laser for excitation of the AlexaFluor488 and reflectance. Images were adjusted for brightness and contrast in ImageJ for presentation purposes.

RESULTS

Time Course Of Activation Of The Cells By Heating

The time course of activation of the cells by heating with a water bath is reported in Figure 2. Typically, no activation was observed for heating durations in the water bath equal to or less than 5min.

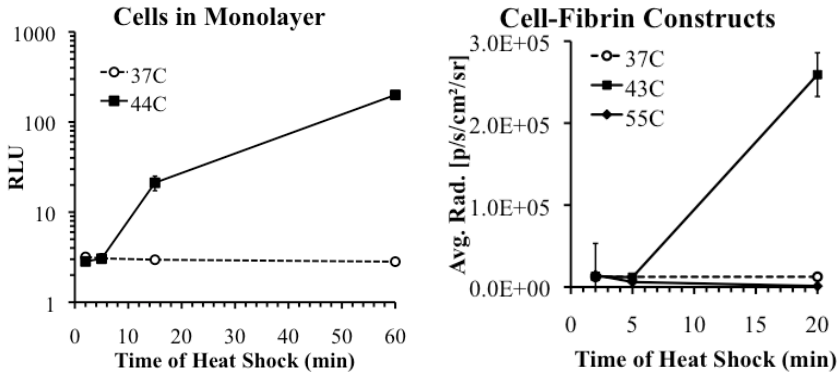

FIGURE 2. Activation of fLuc by exposure to hyperthermia for various durations. Cell monolayers (left) or cell-fibrin constructs (right) were heated on a water bath for 2-60min and the resulting fLuc activity was measured by biochemical assay (monolayer cultures) or bioluminescent imaging (constructs). Only exposures >5min of mild hyperthermia activated expression of fLuc in either culture condition. Highly hyperthermic exposures (T=55C) failed to activate the cells because of cell death. Data are mean ± standard deviation, n = 3.

Cell Activation By HIFU And Influence Of Exposure Conditions

When the cell-fibrin constructs were exposed for 20min to a HIFU intensity of 121W/cm^2, a focal area of activation was observed (Fig. 3, left). When the intensity was increased, a central zone of lower activation appeared. This "biomodal" response is similar to that observed by Kruse, *et al.* in the skin of hsp70b-fLuc transgenic mice exposed to HIFU (Ref 6). We speculate that the thermal dose at the focus was then high enough to induce cell death. Interestingly, high intensity exposures (280W/cm^2) triggered gene expression for exposure times as low as 2min, much shorter than required with water-bath heating (Fig. 3, right).

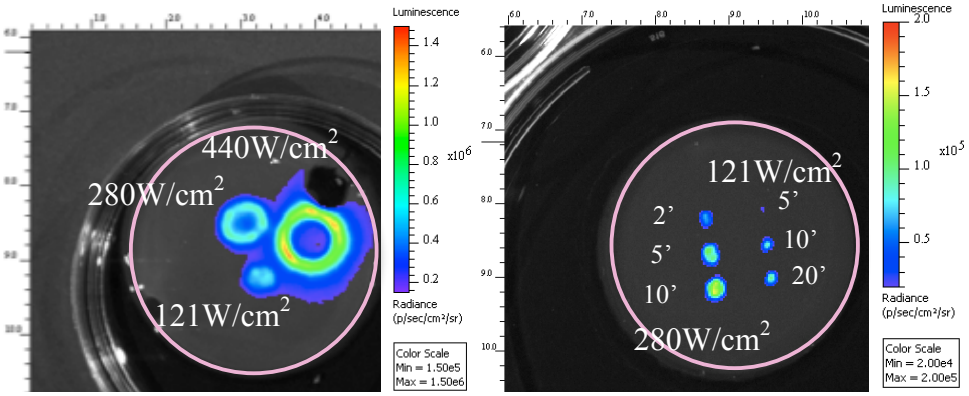

FIGURE 3. Left: Intensity of bioluminescence after 20min of focused ultrasound exposure at different intensities. Right: Intensity of bioluminescence for different exposure durations at intensities of 121 W/cm^2 or 280W/cm^2. The line indicates the perimeter of the cell-fibrin constructs.

Effects Of HIFU On The Morphology Of The Cells And Scaffold

We investigated the effects of focused ultrasound on cell shape, expression of the cytoskeletal protein actin, and morphology of the scaffold. Confocal microscopy revealed a uniform distribution of cells throughout the scaffold, and the cells generally

had a rounded morphology as indicated by the actin staining (Fig. 4, Non-Irradiated). In addition, the fibrin scaffold gave a weak and diffuse, but uniform, signal from reflected light. Following exposure with 121W/cm^2 or 280W/cm^2 focused ultrasound, we observed no substantial differences in the distribution, morphology, or actin expression of the cells; nor was the scaffold altered at these intensities. In contrast, the highest intensity of focused tested, 440W/cm^2, abolished expression of actin within a scallop-shaped region subjacent to the incident surface. In addition, the scaffold appeared to have higher reflectance (Fig. 4, arrowhead), consistent with the directly observable lesion at this focal region (not shown).

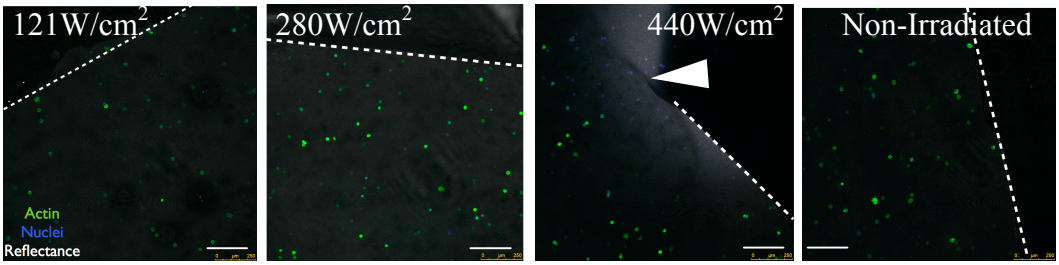

FIGURE 4. Microscopic features of cell-fibrin scaffolds 1 day after 20min focused ultrasound exposures at 121W/cm^2, 280W/cm^2, and 440W/cm^2. A non-irradiated control region is shown at far right. Images are cross sections through the constructs at the focus of each exposure. The incident surface is marked by a dashed line. Staining for the actin cytoskeleton is in green, the nuclear stain is in blue, and reflected light (predominantly from the microstructure of the scaffold) is in white. Arrowhead indicates a disruption at the surface associated with an ultrasound-induced lesion. Scale bars are 250μm.

DISCUSSION

We have demonstrated for the first time the potential for focused ultrasound to trigger a specific, spatially-restricted response in an engineered tissue. The cells used in these studies were modified to express a reporter molecule under the control of a heat shock promoter, and we have identified exposure parameters capable of activating this "gene switch" with little-to-no damage to the cells or scaffolding material.

Other groups have studied the effects of focused ultrasound on the skin and internal organs of transgenic mice harboring the hsp70b-fLuc reporter [4-5]. In those studies, increased expression of fLuc was detected for substantially shorter ultrasound exposure times – as little as 1s at 100W/cm^2 – than in our experiments. We speculate that this discrepancy arises from the differences in acoustic attenuation between native tissues and engineered tissues. The protein content, and perhaps most importantly the collagen content, of native tissues is at least 10-fold higher than that in our cell-fibrin constructs. Given the well-established correlation between protein content and acoustic attenuation of soft tissues [6], we expect that our engineered tissues absorb less acoustic energy than the tissues of a mouse and thus require higher energy inputs for activation of the hsp70b-fLuc. Differences in cell density between native and engineered tissues and between other aspects of the experimental setups (*e.g.*, transducer) may also contribute to the marked differences in the exposures required to trigger expression of fLuc.

Another interesting finding from our studies was that activation of hsp70B-fLuc by focused ultrasound occurred more rapidly than in control experiments in which engineered tissues were simply heated with a hyperthermic bath. Whereas bath hyperthermia at 43-44C consistently required more than 5min to increase fLuc activity, we observed activity after as little as 2min of ultrasound exposure. This result suggests that non-thermal effects of the ultrasound exposure contribute to the stress response that drives expression of fLuc in these cells, and ongoing studies are addressing this possibility.

These studies lay the groundwork for using focused ultrasound to stimulate the expression of specific therapeutic molecules such as BMP-2 and VEGF. As with the spatially-restricted activation of fLuc, we envision applying focused ultrasound to cell populations modified to express one or more of these growth factors under the control of the hsp70b promoter. This approach would provide spatial and temporal control over the expression of factors known to stimulate bone formation and may thus be useful in the *ex vivo* fabrication of engineered bone grafts and the augmentation of bone regeneration *in vivo*.

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